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Neuroantibodies: Ectopic Expression of a Recombinant Anti-Substance P Antibody in the Central Nervous System of Transgenic Mice

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Summary

Recombinant antibodies are efficiently secreted by cells of the nervous system. Thus, their local expression in the CNS of transgenic mice could be used to perturb the function of the corresponding antigen. As a first application of this approach, we have generated transgenic mice that express antibodies against the neuropeptide substance P, under the transcriptional control of the promoter of the neuronal gene *vgf*. The transgenic antibodies are expressed in a tissue-specific and developmentally regulated manner and are effective in competing with the endogenous substance P, as demonstrated by a marked inhibition of neurogenic inflammation and by motor deficits. This phenotypic knockout approach may provide a complementary alternative to gene knockout by homologous recombination.

Introduction

Substance P (SP), a neuropeptide of the tachykinin family, exerts several biological activities in the CNS and in peripheral organs, mainly, but not exclusively, as a neurotransmitter and neuromodulator (reviewed in Otsuka and Yoshioka, 1993). The functional roles of the SP neuropeptide in the mammalian CNS and PNS are currently being investigated by a variety of experimental approaches, including the use of antibodies and of pharmacological agonists and antagonists. However, a continuous delivery of antibodies or pharmacological drugs to the nervous tissue poses several practical problems, mainly due to limited diffusion and half-life of the injected substance. Moreover, the long-term use of pharmacological agonists and antagonists in vivo is also limited by secondary nonspecific effects.

Following the demonstration that cells of the nervous system are very efficient in the secretion of recombinant antibodies (Cattaneo and Neuberger, 1987), we have developed an experimental approach (neuroantibodies) for functional and developmental studies in the nervous system, based on the ectopic expression of recombinant antibodies by cells of the nervous system itself (Piccioli et al., 1991), achieved by a suitable choice of transcriptional regulation sequences to direct the expression of an antibody of interest.

This strategy was applied to the study of the neuropeptide SP, as this neuropeptide has multiple sites of action in both the PNS and CNS. We took advantage of the rat monoclonal antibody NC1/34HL (Cuello et al., 1979), which recognizes with a high affinity the amidated C-terminal portion of the SP peptide responsible for receptor binding, and does not cross-react with the related tachykinin peptides neurokinin A (NKA) and neurokinin B (NKB). In this paper we present the results concerning the generation and the study of transgenic mice that express recombinant anti-SP antibodies (Piccioli et al., 1991) under the control of the promoter of the neuronal *vgf* gene (Levi et al., 1985), which encodes for a protein that is induced in vitro by nerve growth factor (NGF) in PC12 cells (Levi et al., 1985) and is stored in secretory granules of cells of neuronal and endocrine origin (Possenti et al., 1989). VGF RNA and protein are expressed in vivo in discrete cell populations of the CNS and PNS, as well as in the adrenal medulla (van den Pol et al., 1989, 1994; Ferri et al., 1992; Salton et al., 1991). Many of these regions show SP and/or NK1 receptor expression as well. We show here that the recombinant antibodies are expressed in both the PNS and CNS, with a postnatal spatiotemporal pattern of expression that parallels that of the endogenous *vgf* gene. The phenotypic effects caused in adult mice by the expression of transgenic antibodies were assessed by functional assays related to the SP peptidergic system, including acute nociception, neurogenic inflammation, and motor and exploratory behavior, and confirm that the neutralization of SP action is effective in this mouse model. This report proves that the neuroantibody approach (Cattaneo and Neuberger, 1987; Cattaneo et al., 1989, Soc. Neurosci., abstract; Piccioli et al., 1991) can be utilized to interfere in vivo with the action of a target neuronal antigen. The results obtained provide a valuable model for the study of SP actions and of *vgf* transcriptional regulation. Moreover, these results suggest that the neuroantibody approach may be useful for studying gene function in the CNS, as a complementary alternative to gene knockout by homologous recombination. In particular, this approach should prove valuable for those genes, such as that coding for preprotachykinin, whose expression is regulated in a subtle way by alternate splicing, yielding more than one bioactive peptide.

Results

Production and mRNA Analysis of Transgenic Mice Expressing Anti-SP Antibodies under the Transcriptional Control of the *vgf* Promoter

The rat monoclonal antibody NC1/34HL (Cuello et al., 1979) is directed against the amidated C-terminal portion of the SP neuropeptide, which is responsible for receptor binding (Otsuka and Yoshioka, 1993). This antibody is therefore effective in inhibiting the binding of SP to its receptors and the ensuing biological actions (Bannon et al., 1983). Moreover, NC1/34HL does not cross-react with the related neurokinin peptides NKA and NKB, which differ from SP by only one residue in the C-terminal pentapeptide. With the aim of neutralizing SP action in vivo by the neuroantibody strategy, the variable regions of the antibody have been previously cloned and reassembled with human constant regions instead of the original ones, to facilitate the subsequent detection of the transgenic antibody chains against the background of mouse immunoglobulins (Piccioli et al., 1991). For the present study, we selected the promoter of the rat *vgf* gene (Levi et al., 1985) to direct antibody expression in the nervous system of transgenic mice. A 1.5 kb DNA region 5′ to the initial methionine (hereafter referred to as the "*vgf* promoter") directs a strong expression of a reporter gene in neuronal cultured cells (Possenti et al., 1992). The heavy and light chains of the recombinant NC1 anti-SP antibody were placed under the transcriptional control of the *vgf* promoter region in separate plasmids (Figures 1A and 1B), and the transcriptional efficiency of these plasmids was confirmed by transfection in nonlymphoid cells permissive for this promoter, such as PC12 (pheochromocytoma) cells and GH3 (pituitary) cells.

For the production of *vgf* anti-SP transgenic mice, the linearized DNA from both plasmids (Figures 1A and 1B) was coinjected into the pronucleus of fertilized mouse eggs; 33 mice were born after egg implantation. Of these, 4 had DNA for both chains integrated, and 1 had the light chain transgene only (founder 8507). Of the double transgenics, 2 did not transmit the transgenes to offspring, and 1 (founder 8530) expressed the transgenes at lower levels. Therefore, we concentrated our studies on the double-positive (8510) and single-positive (8507) founders, but

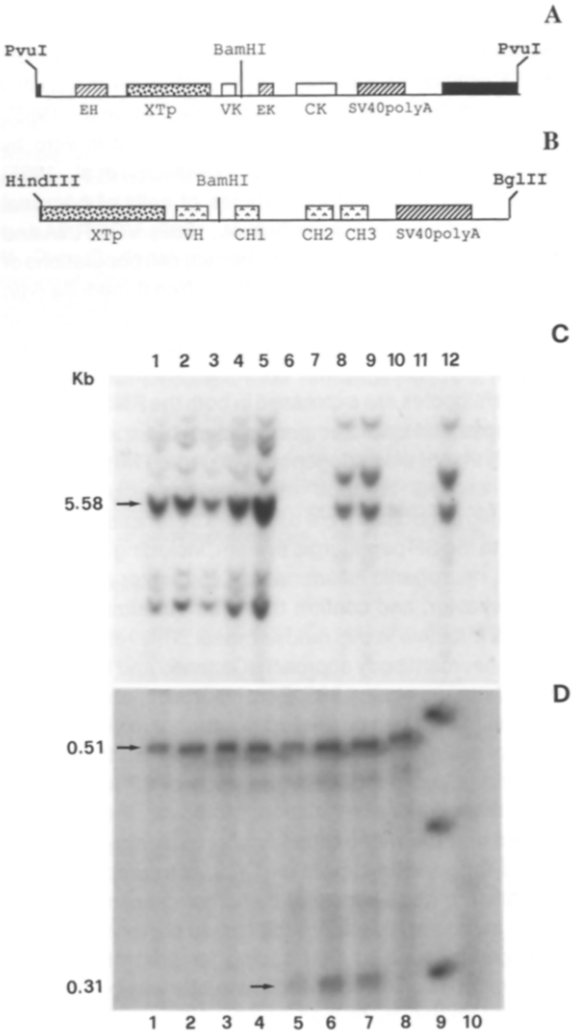

Figure 1. Production and mRNA Analysis of Transgenic Mice Expressing Anti-SP Antibodies

(A and B) DNA constructs used for the production of the transgenic mice. Light chain (A) and heavy chain (B) transgenes were used to generate transgenic mice by microinjection in fertilized 1-cell eggs. Linearized DNA was prepared by digestion with PvuI (light chain) and HindIII–BglII (heavy chain) of pSVhygro-VGF-V_κSP and pSVgpt-VGF-V_HSP, respectively (Piccioli et al., 1991). CK and CH1–CH3, human constant region domains of the light (κ) and heavy (γ1) chains; EH and EK, enhancers of the immunoglobulin heavy and light chain genes, respectively; V_κ and V_H, light and heavy chain variable regions of the NC1/34HL monoclonal antibody; XTp, 5′ region of the *vgf* gene (from −806 to +693 of the transcription initiation start), encompassing the putative promoter region and a spliced intron just upstream of the initiating methionine codon (Possenti et al., 1992); the closed boxes in the light chain construct refer to vector sequences. Scale, 1 cm = 1000 bp (A) or 400 bp (B).

(C) Southern blot analysis of F1 and F2 progeny of transgenic mice derived from founder 8510 (lanes 1–7) and founder 8530 (lanes 8–12). DNA (20 μg) was digested with BamHI and hybridized using the 3 kb BamHI–BglII fragment (Cγ probe; see [B]) as a probe. Results for the heavy chain transgene are shown. A 5.6 kb band lights up, corresponding to head-to-tail insertions of multiple copies of transgenes.

(D) RNase protection analysis of the transgenic antibody chains. Results for the light chain are shown. Total RNA was extracted from brains of adult wild-type (lanes 4 and 8) and *vgf* transgenic (lanes 1–3 and 5–7) mice. Total RNA (20 μg) was hybridized to a ^{32}P-labeled antisense RNA probe specific for the 3′ terminal end of the rat *vgf* gene in the absence (lanes 1–4) or presence (lanes 5–8) of a V_κ-specific antisense RNA probe. A 510 bp protected fragment, specific for the endogenous *vgf* gene, is observed in transgenic (lanes 1–3 and 5–7) and wild-type (lanes 4 and 8) mice, as expected, while a protected band corresponding to the transcript from the V_κ transgene is evident in the brain of transgenic mice (lanes 5–7). Numbers on the left represent molecular size (in kilobases) as derived from a ^{32}P-labeled pBR322-MspI marker (lane 9); *vgf* and V_κ RNA probes digested in the absence of added RNA are also shown (lane 10).

results on the expression pattern of the transgene were also confirmed on mice from family 8530. Homozygous transgenic mice obtained after breeding are viable, but they mate and reproduce at a greatly reduced rate. Although this effect has not been systematically quantitated, it limited significantly the number of mice available for experimental study with respect to their single transgenic or nontransgenic counterparts. This phenotypic effect is consistent with reports that neonatal capsaicin treatment leads to a marked reduction of the mating and reproduction rate of both male and female rats, presumably a sensory fiber–mediated phenomenon (Traurig et al., 1984). In a separate set of microinjections, transgenic mice were derived with the same transgenic antibody under the transcriptional control of the immunoglobulin heavy chain promoter (P. P. and A. C., unpublished data). One of the families derived (family 8) was utilized for some of the experiments described below.

Figure 1C shows a Southern blot for the heavy chains on DNA samples from individual mice deriving from founders 8510 and 8530 (both double-positive transgenics), which confirms that an identical restriction pattern is obtained for the mice within each group and shows that more than one copy of each transgene is integrated, in a head-to-tail arrangement.

The expression of the transgenes in different organs of adult mice was first verified at the mRNA level, by RNase protection analysis. Figure 1D shows that the mRNA for the NC1/34HL light chain is expressed in the brain of adult transgenic mice (Figure 1D, lower arrow). The levels of mRNA for the transgenic antibody chain appear to be lower than those of the abundant endogenous *vgf* mRNA (Figure 1D, upper arrow), suggesting that sequences in the 3′ or 5′ untranslated regions of the *vgf* gene, not present in our construct, may contribute to increased mRNA levels, possibly by increasing its transcription or its stability. RNase protection analysis of different tissues showed that V_κ and *vgf* mRNAs were detected in brain, but not in kidney, liver, spleen, and total adrenal glands (data not shown). mRNA for the transgenic light chain was detectable in cerebellum and spinal cord at low levels, while *vgf* transcripts could be more easily detected there (data not shown). Qualitatively similar results were obtained for the heavy chain mRNA, although the levels of mRNA appear to be lower. This is most likely due to secondary structure of the V_H mRNA and does not reflect a genuine difference in the levels of mRNA for the light and heavy chains, as this difference is also found with the mRNA isolated from the NC1 hybridoma cell line (data not shown) and as the heavy chain protein can be very easily detected (see below).

The postnatal expression of antibody chain mRNA in the brain, which was confirmed in litters from 2 independent founders (8510 and 8530), peaks around P10–P12 and declines thereafter (data not shown), in very close agreement with the expression of *vgf* mRNA (Salton et al., 1991).

In conclusion, the expression of the transgene mRNA(s), transcribed from the *vgf* promoter, appears to parallel that of the endogenous *vgf* gene.

Functional Anti-SP Antibodies Are Synthesized in the Brain and in the Serum of Transgenic Mice

We then asked whether the chimeric anti-SP antibodies are functional. As the binding of NC1/34HL to SP requires both the heavy and light chains and the isolated NC1/34HL chains alone do not retain SP-binding activity (Piccioli et al., 1991), functional antibodies can be made only if the two antibody chains are coexpressed in the same cells. Heavy and light chain assembly cannot occur extracellularly to any significant extent, and moreover, the heavy chain alone cannot be secreted in the absence of a light chain.

The presence of transgenic SP-binding antibodies in the serum was determined at different postnatal ages, with an enzyme-linked immunosorbent assay (ELISA) on solid phase–coupled SP peptide (Figure 2A). Two main conclusions can be drawn from this experiment. First, SP-binding antibodies can indeed be detected in the serum, demonstrating assembly (and therefore coexpression) of the two antibody chains. These antibodies are most likely produced in endocrine tissues, such as the adrenal medulla and the pituitary, where the *vgf* gene is expressed (Ferri et al., 1992). Second, the level of SP-binding transgenic antibodies in the serum appears to be developmentally regulated, increasing after birth up to a maximum value around the second to third postnatal week, after which it falls down. This is consistent with the developmental time course seen at the mRNA level (see above) and was further confirmed by the immunocytochemical analysis described below. The serum of adult mice (2–10 months old, families 8510 and 8) was shown not to contain mouse immunoglobulins directed against human transgenic antibodies (data not shown).

The amount of recombinant antibody found in the serum was also quantitated by incubation with ^{125}I-labeled SP peptide (Figure 2B). This yields an average value of 60 ng/ml for the concentration of chimeric SP antibodies in the serum of 3-week-old transgenic mice, as determined by comparison to a calibration curve with purified NC1/34HL antibody. In the adult, these levels fall to 6 ng/ml. The levels determined in the serum for the *vgf* anti-SP mice are comparable to those found in the serum of family 8 mice (~2.5 ng/ml). Negative controls in these assays included serum samples from the family 8507 transgenic mice, which express only the light chain. The binding of ^{125}I–SP to transgenic antibodies is competed by a molar excess of unlabeled SP (data not shown), further confirming the specificity of the binding.

This assay was used to demonstrate the presence of SP-binding antibodies in different brain regions at the ages of P21 (Figure 2B) and 3 months (Figure 2C), thus confirming that coexpression of heavy and light chains, to yield a functional antibody, is indeed occurring in cells of the nervous system. At 3 weeks, the highest levels of antibodies are found in the cortex and hippocampus, followed by pons, hypothalamus, and cerebellum (Figure 2B). Interestingly, antibodies are also present in the adrenal gland, in keeping with the expression of *vgf* in the adrenal medulla (Ferri et al., 1992). It should be noted that this assay, based on the binding of exogenously added ^{125}I–SP, could under-

estimate the amount of antibody present, owing to the presence of endogenous SP that competes with the antibodies, following tissue extraction. In the adult *vgf* mice, transgenic antibodies can be readily detected in different brain regions, albeit at lower levels (Figure 2C). In 3-month-old family 8 mice (immunoglobulin promoter), anti-SP antibodies can be detected in the serum (~2.5 ng/ml) and in the spinal cord (40 ng/mg S100).

Distribution of Transgenic Anti-SP Antibodies in the CNS

The spatial distribution of anti-SP antibodies in the CNS was studied by immunocytochemistry. The general cytoarchitectonic structure of brain regions did not reveal any obvious alteration in the transgenic mice. The overall picture is that of an abundant staining of many discrete areas throughout the nervous system, with a predominantly, if not exclusive, neuronal expression. Figure 3 and Figure 4 illustrate some examples of the staining obtained for the heavy chain in coronal brain sections derived from transgenic mice at P16. An abundant antibody expression can be seen in the piriform and perirhinal cortex, as well as through the amygdaloid complex (Figure 3A). Figure 3B shows the distribution of the transgenic heavy chain in the hippocampal region. A stronger density of positive cells is evident in the CA1 and CA2 regions, but positive

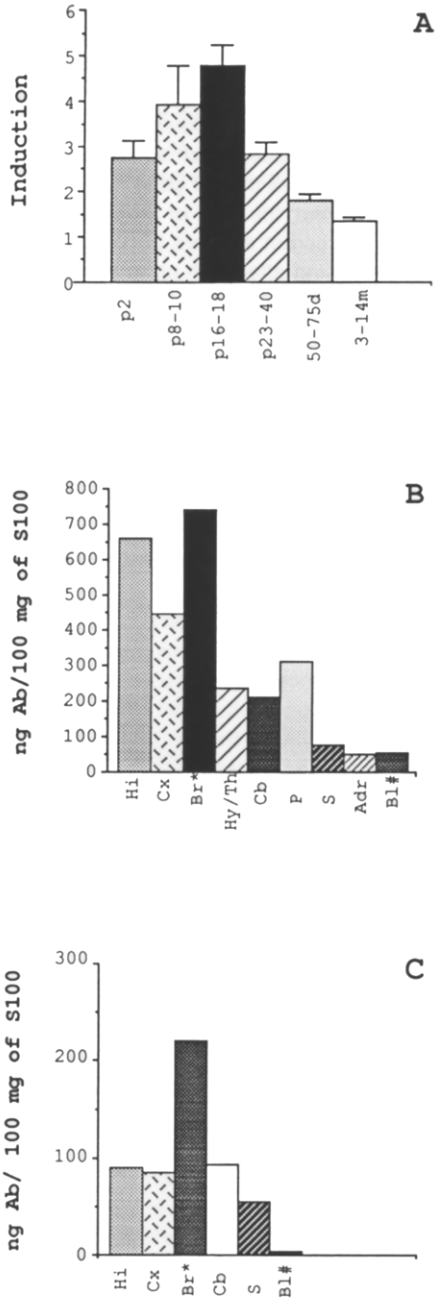

Figure 2. Postnatal Expression of the Recombinant Anti-SP Antibody in the Serum and in Tissues of Transgenic Mice

(A) ELISA on blood samples at different postnatal ages. Blood was collected from transgenic and negative mice, and serum was tested by ELISA for the presence of human antibodies binding to solid-phase SP. Data are reported as fold of induction over the background value (obtained for samples from negative age-matched controls, value equal to 1), calculated as the ratio between the absorbance values for positive and negative mice in each assay. Values reported represent the average of data collected in four separate experiments for adult animals (np = 23, nc = 9); in two experiments for P2 (np = 6, nc = 2), P8–P10 (np = 10, nc = 7), and P16–P18 (np = 15, nc = 4) mice; and in one single experiment for P23–P40 (np = 8, nc = 4) and P50–P75 (np = 11, nc = 9) mice (np, number of double-positive mice tested; nc, number of single-positive and/or negative mice tested). Bars indicate ± SEM.

(B and C) SP-binding antibodies in CNS regions of P20 (B) and adult (C) transgenic mice. Brain regions, spinal cord, and adrenal glands were dissected from P20 and adult (3-month-old) transgenic mice, and the presence of ^{125}I–SP-binding transgenic antibodies was determined (see Experimental Procedures). Data are reported as nanograms of antibody present in 100 mg of pooled S100 extracts (see Experimental Procedures) and represent the results obtained for transgenic mice (n = 6) after subtracting the background values obtained for control mice (n = 5). Each pooled extract was assayed in three to four different experiments, and the differences between each experimental determination were always less than 10%. The amount of antibody present was determined by comparison to titration curves, performed in each experiment, with purified NC1 antibodies. Hi, hippocampus; Cx, cortex; Br*, the rest of the brain, after dissection of the indicated regions; Hy/Th, hypothalamus/thalamus; Cb, cerebellum; P, pons; S, spinal cord; Adr, adrenal gland; Bl#, determination of anti-SP antibodies in blood samples from the same mice (pooled results).

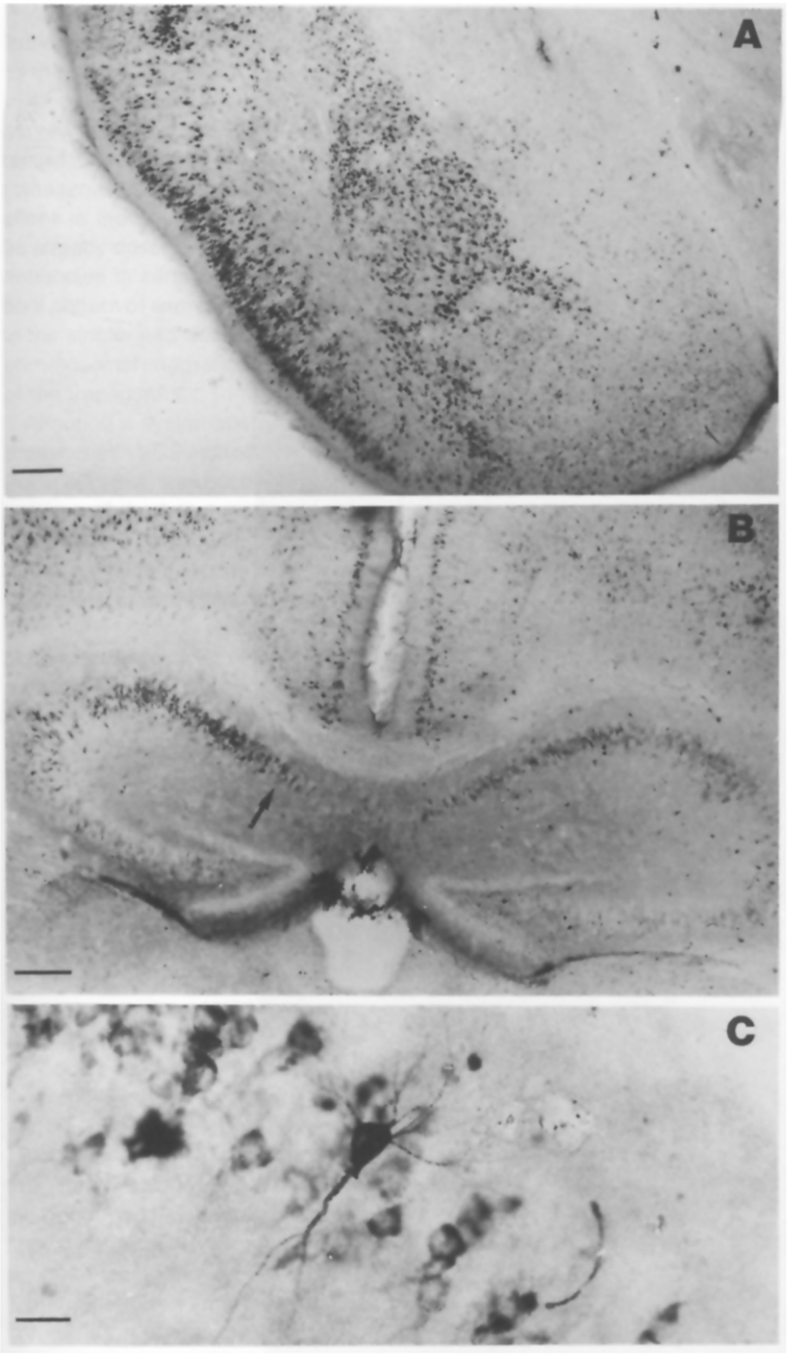

Figure 3. Ectopic Expression of Recombinant Anti-SP Antibody Chains in the CNS of Transgenic Mice

Results for the heavy chain are shown; 50 μm coronal sections of the brain from a double-transgenic mouse sacrificed at P16. Expression of the heavy chain can be seen in the piriform and perirhinal cortex as well as throughout the amygdaloid complex (A), and in the hippocampus (B). A neuron-specific staining can be observed in (C), which shows, at higher magnification, a pyramidal cell of the hippocampal CA1 region (arrow in [B]). Bars, 240 μm (A and B), 24 μm (C).

cells can be found throughout the hippocampal formation. A pyramidal cell of CA1 reacting positively for the heavy chain is shown in Figure 3C. The high levels of transgenic antibodies found in the hippocampus are particularly noteworthy in view of the fact that SP and SPergic nerve terminals are also abundantly present in the hippocampus, subserving a functional role that is still unclear (Leranth and Nitsch, 1994). Among other brain regions, all cortical areas (Figures 4A and 4B) as well as olfactory nuclei and tubercles also show good expression of the transgenic antibodies, while thalamic nuclei show a lower density of strongly reactive cells (e.g., Figure 4D). Spinal cord, where SP plays important roles, also shows positively staining neurons (Figures 4E and 4F). A high density of strongly reacting neurons is also found in other brain regions, such as the central gray and reticular formations, while striatum, caudate putamen, and hypothalamus show scattered positively staining neurons. Of other CNS regions where SP is present at high levels, substantia nigra proved to be consistently devoid of antibody expression. It remains to

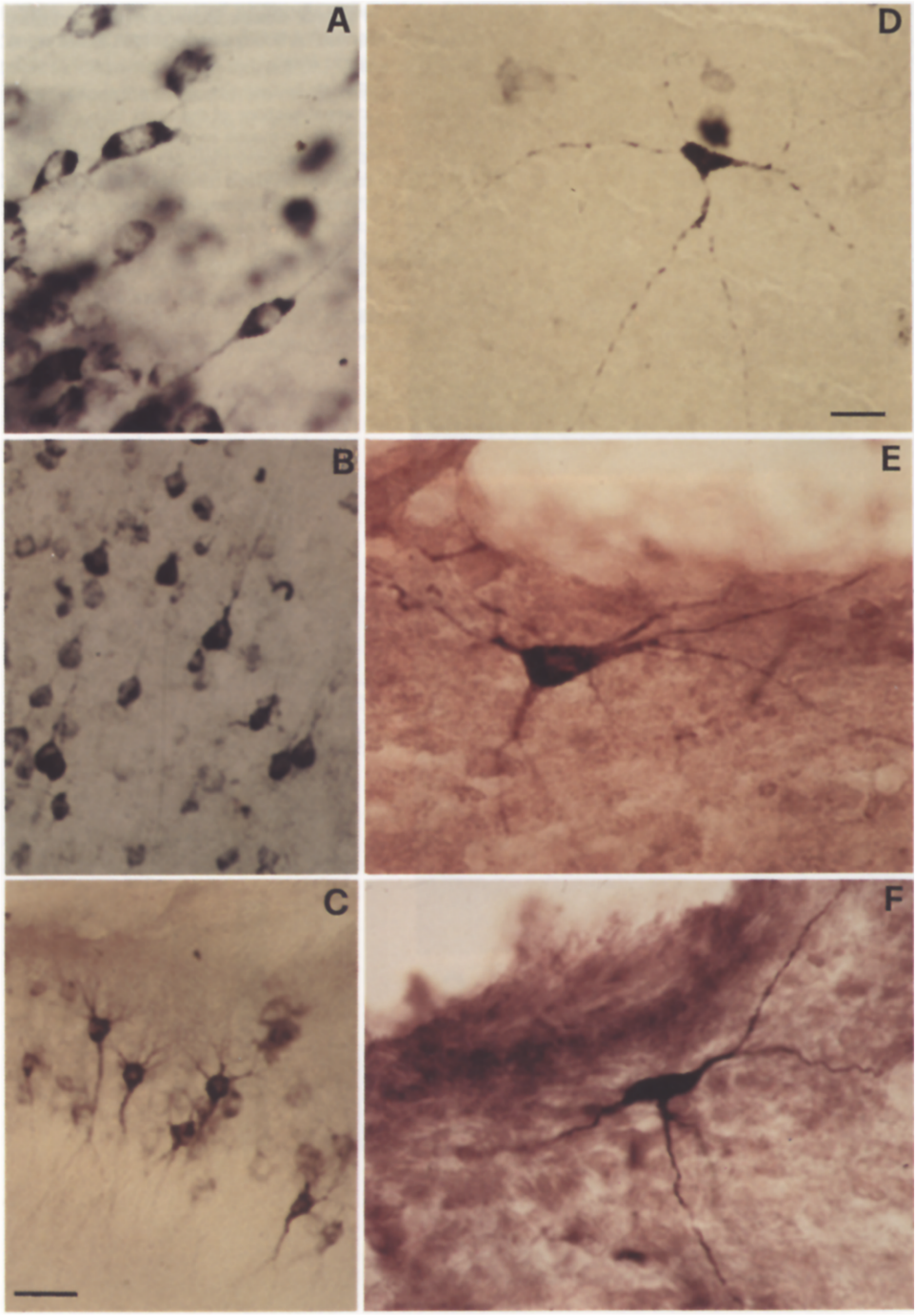

Figure 4. Types of Neuronal Cells Expressing Antibody Heavy Chains in the Nervous System of Transgenic Mice

(A) Bipolar cells of the piriform cortex; (B) pyramidal neurons of the frontal cortex (magnification of [B]); (C) pyramidal cells from the CA2–CA3 hippocampal fields (sagittal section); (D) multipolar thalamic neuron; (E and F) scattered neurons surrounding the ependymal canal in thoracic spinal cord. A punctate staining is evident in this case, probably reflecting secretory vescicles. Bars, 24 μm (A, D, E, and F), 48 μm (B and C).

be verified by radioimmunoassay whether antibodies released by nearby regions are present in the striatonigral system.

Inspection at a higher magnification shows that the staining is mainly, if not exclusively, neuronal, with neurons of many different morphologies being found in different brain regions (Figure 4; neurons from piriform cortex [A], frontal cortex [B], CA2 field of the hippocampus [C], thalamus [D], and spinal cord [E and F]). These pictures show that the staining is distributed throughout the cell extension, including its cellular processes and arborization. It will be extremely interesting to analyze with electron

microscopic immunochemistry the fine intracellular distribution of the transgenic antibodies, with particular regard to the synaptic regions.

A comparison of the immunocytochemical staining observed in sections of P16 transgenic mice with that obtained for adult mice confirmed that the expression of the transgenic antibody is higher in the former case and declines in the adult (in terms of number of labeled cells), as already described above for the titer of the transgenic antibodies in serum. A very similar, almost superimposable pattern of expression is found for the light chain, both in the single- and double-transgenic mice. This rules out chromosomal integration effects on the expression pattern of the transgenes.

Although a systematic comparison of the antibody expression with VGF expression in the mouse brain (van den Pol et al., 1994) was not performed, the overall expression patterns of the two genes are remarkably similar, which supports the conclusion that the 1.5 kb promoter region of the *vgf* gene contains most (if not all) of the elements necessary to determine its tissue specificity. Thus, the transgenic antibody chains can be considered as reporter genes for the activity of *vgf* regulatory sequences. This mouse model can therefore be used to address the question of the sensitivity of *vgf* gene regulatory sequences to induction by NGF, by other neurotrophins, and by electrical activity in vivo (Lombardo et al., 1994).

Quantitative and qualitative differences between the expression patterns of the transgenic antibodies and the endogenous *vgf* gene, however, can be seen. These could be ascribed to the lack of some other unidentified regulatory element(s) in the transgene construct utilized; to the fact that the expression of endogenous *vgf* has been studied, in the published reports, after colchicine treatment, to facilitate the detection of VGF immunoreactivity in cell bodies (van den Pol et al., 1989, 1994); to effects of the anti-SP antibodies on the expression of the *vgf* gene itself; or to differences between *vgf* expression in the mouse and in the rat. This will require further investigation.

Functional Studies Related to the SP System

We performed functional experiments to ask whether antibody expression is efficient in competing some of the action(s) of endogenous SP.

In the periphery, there is good evidence that SP is involved in the process of neurogenic inflammation (Lembeck and Holzer, 1979; Lembeck et al., 1992). Its role in nociception has been controversial (Urban et al., 1994), possibly being only one of several transmitters. In the CNS, SP has been implicated in a variety of functions (Otsuka and Yoshioka, 1993), including control of motor activities through a net excitatory input of SP in the striatonigral system and a potential neuroendocrine role of hypothalamic SP. In these mice, we have tested acute nociceptive behavior (tail immersion and hot plate test), neurogenic inflammation upon mustard oil application on the skin, and motor activity and exploratory behavior in the open field test. For practical reasons, these functional tests were performed on adult (3-month-old) mice, even if the levels of the antibody are lower in adult than in younger animals (Figures 2B and 2C).

The reaction times in the hot plate test and the tail withdrawal latencies did not differ in anti-SP transgenic mice with respect to age-matched controls (Figure 5A). On the contrary, mustard oil–induced plasma protein extravasation was significantly reduced in mice expressing the anti-SP antibody (65% reduction; Figure 5B, part I). Tests for motor activity were also performed, as a simple inspection of the transgenic mice showed that they were quiter and less scared upon handling, allowing blind observers to distinguish them from their normal or single-transgenic littermates. Consistent with this observation, a decreased locomotor activity was demonstrated for the transgenic mice in the open field test, with a very significant reduction of line crossings and rearings (Figure 5C, part I).

The inhibition of neurogenic inflammation by anti-SP transgenic antibodies was confirmed on another independent line of transgenic mice (family 8; Figure 5B, part II). The expression of the anti-SP antibodies in these mice is directed by the immunoglobulin promoter, and the resulting levels in the serum are comparable to those of age-matched mice from family 8510, as determined by binding of ^{125}I–SP. Also in mice from family 8, the plasma extravasation following neurogenic inflammation was inhibited close to 40%. On the other hand, family 8 mice did not show the decrease in motor activity observed for the *vgf* anti-SP mice (Figure 5C, part II), consistent with the expected reduced expression of the transgenic antibodies in the CNS of these mice. Preliminary observations did not point toward any effect on acute nociceptive behavior in these mice.

Altogether, these results show that the neutralization of the SP peptide by the expression of recombinant antibodies is functionally effective.

It was of interest to determine whether the expression of the transgenic antibodies affects the levels of the endogenous SP peptide. Indeed, it is expected that inhibition of SP activity in vivo should occur by a competitive effect at the level of peptide binding to the receptors, and not by an effect on the level of the peptide itself. SP levels were determined by radioimmunoassay in brain samples from negative and transgenic mice (Table 1). For midbrain and medulla, it was confirmed, as initially anticipated, that SP levels in transgenic mice were identical to those in controls. Surprisingly, in spinal cord and sciatic nerve, a small but significant increase of SP levels was observed in transgenic mice. We do not know at this stage whether this feedback mechanism is activated at the transcriptional, translational, or posttranslational level, or whether it involves other neuropeptides and/or transmitters as well. The expression of the preprotachykinin mRNA in the striatum of *vgf* anti-SP mice was shown not to differ from those of control mice (data not shown). Also, immunohistochemistry of the SP peptide itself throughout the CNS did not show any gross difference with the controls (data not shown). Future studies will be needed to clarify further this putative feedback mechanism uncovered by the action of the transgenic antibodies.

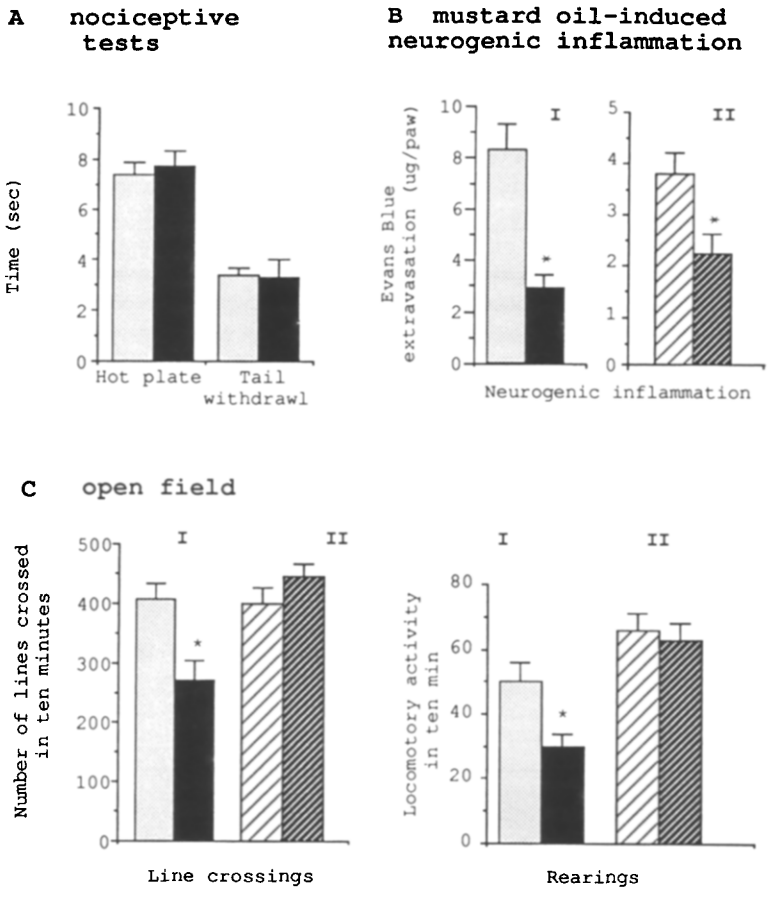

Figure 5. Functional Tests of SP Action

Thermonociception (A) was evaluated by the reaction time in the hot plate test and by the tail withdrawal latency from 50°C water (stippled bars, control [n = 8]; closed bars, family 8510 [n = 7]). Neurogenic plasma protein extravasation (B) was evoked in the hind paw by challenging with 5% mustard oil in paraffin oil (part I, family 8510; part II, family 8; see Results). Motor activity (C) was evaluated in the open field test by counting line crossings and rearings during a 10 min observation period. Data represent means ± SEM (asterisk, $p \leq .05$).

In (B), n = 7 for family 8510 (closed bars) and family 8 (gray hatched bars), and n = 8 (stippled bars) and n = 15 (white hatched bars) for control mice of the appropriate strain for the neurogenic inflammation test. In (C), n = 7 for family 8510 (closed bars), n = 10 for family 8 (gray hatched bars), and n = 8 for control mice in the locomotor activity tests (stippled bars and white hatched bars).

Discussion

Much can be learned in the study of the mammalian nervous system by using methods to interfere in a specific manner with the action of selected molecules in vivo. Of the methods introduced so far for the mouse nervous system, gene knockout by homologous recombination has proved an extremely successful and powerful method for generating mice carrying predesigned mutations in the germline (Capecchi, 1989). However, because the mutation, most commonly a null mutation, is introduced in the germline of the mutant animals, it will exert its effects from the onset of animal development. Although this approach to gene inactivation is valuable, for many applications it is important that the inactivation of a particular gene occurs in a conditional or partial manner (e.g., in a predefined cell lineage or tissue, or at a certain stage of development), and in particular in the adult organism. This would allow one to overcome problems posed by the fact that null mutations in the germline are often lethal, and would also allow a more precise and unambiguous analysis of the impact of a mutation. While conditional gene targeting now seems feasible, through the use of the Cre–*loxP* recombination system (Gu et al., 1993), we have taken a different approach (the neuroantibody approach; Piccioli et al., 1991), building on work with the expression of recombinant antibodies in mammalian nonlymphoid cells, as secreted (Cattaneo and Neuberger, 1987) or intracellular (Biocca et al., 1990; Biocca and Cattaneo, 1995) proteins. Our approach is based on the expression of recombinant versions of monoclonal antibodies by cells of the nervous system in transgenic mice, under the transcriptional control of suitable promoters. The local expression of secreted antibodies could be used to inactivate a selected gene product active in the extracellular environment of the nervous system of transgenic mice.

Here we report the application of this experimental strategy to the study of the tachykinin neuropeptide SP. The tachykinin gene encodes for multiple peptides and is regulated by tissue-specific alternative splicing. A gene knockout approach to disrupt the SP-coding region of this gene would have to be designed to preserve the expression of the other peptides and the correct splicing of the rest of the RNA molecule; this would be technically demanding.

Table 1. SP Level in Tissue of Negative and Transgenic Mice

Tissue	Negative (n)	Transgenic (n)
Midbrain	2915 ± 274 (11)	3174 ± 236 (10)
Medulla/brainstem	3896 ± 321 (12)	4090 ± 225 (10)
Lumbar spinal cord	2245 ± 114 (6)	2769 ± 137 (5)[a]
Sciatic nerve	371 ± 13 (12)	544 ± 31 (10)[b]

SP levels were determined by radioimmunoassay and are presented in femtomoles of protein per milligram of tissue (± SEM).

[a] $p < .05$.

[b] $p < .01$.

For this reason, we consider this a case for which the neuroantibody approach may be convenient, exploiting the NC1/34HL monoclonal antibody, which recognizes only the SP peptide and not the related peptides. We have generated transgenic mice that express recombinant antibodies directed against the neuropeptide SP, under the transcriptional control of the promoter of the neuronal *vgf* gene, and have shown that the transgenic antibodies, expressed in a tissue-specific and developmentally regulated manner, are indeed able to compete with some biological actions of the endogenous SP.

Neurokinins are a group of structurally related peptides characterized by a common C-terminal pentapeptide that interact with different receptor subtypes. The neuropeptide SP has multiple actions in the nervous system, some of which have been unequivocally demonstrated, while others are more controversial or totally unknown. For instance, SP has been strongly implicated in the transmission of pain, being (co)released, alongside other transmitters and peptides, from small-diameter primary afferents and acting primarily on NK1 receptors in the spinal cord. However, the involvement of SP as the primary neurotransmitter in acute nociception has been questioned on the basis of studies with pharmacological antagonists. On the other hand, the primary role of SP in neurogenic inflammation, smooth muscle contraction, and vasodilation is rather well established. As for the CNS, where the SP peptide and its receptor are widely distributed, a functional role(s) is far from clear. The study of the physiological effects of neurokinins with respect to neurokinin receptor subtypes is hampered by the fact that the endogenous neurokinins are themselves preferential, but not selective, agonists. This is why competing directly with the ligand (as we do in our approach) rather than with receptor antagonists may be preferable. Much work has been performed with pharmacological antagonists, but studies in this field have been limited by the lack of efficacious antagonists specific for the NK1 receptors, and even more by the difficulty of using these antagonists in vivo, owing to administration route, short half-life, and side effects. It should also be noted that pharmacological antagonists are ideal for use in acute preparations, while they are not suited for chronic inhibition studies. Our transgenic model goes some way in the direction of achieving a more prolonged inhibition of the SP neuropeptide, during and after the development of the nervous system. This will allow us to study not only the effects of the acute block of the peptide, but also the modulatory consequences of a more prolonged inhibition of the peptide action on other related systems. For example, it will be interesting to utilize this mouse model to ask questions related to the development of SPergic synaptic pathways under conditions in which the action of the peptide is (constantly, albeit partially) inhibited. In addition, the recently proposed role of SP as a modulator of chemoattractants for commissural axons in the spinal cord floor plate (De Felipe et al., 1995) will be ideally studied in this mouse model.

The phenotypic effects caused in adult mice by the expression of transgenic antibodies were assessed by behavioral assays related to the SP peptidergic system and confirmed that the neutralization of SP action is effective in this mouse model, as deduced from the results obtained on neurogenic inflammation, which is known to be mediated by SP and NK1 receptors (Lembeck and Holzer, 1979; Lembeck et al., 1992). The inhibition of neurogenic inflammation by transgenic anti-SP antibodies was observed in 2 independent lines of transgenic mice, allowing us to rule out chromosomal integration effects. The lack of inhibition in the acute nociceptive behavioral test is consistent with the lack of effect of recently introduced nonpeptide NK1 antagonists in this phenomenon (Courteix et al., 1993; Fleetwoodwalker et al., 1993; Garces et al., 1993; Yamamoto and Yaksh, 1992; Yamamoto et al., 1993). As the role of neurokinin peptides is most likely linked to the complex dynamic alterations in nociceptive pathways occurring in chronic pain (Dray et al., 1994), it is anticipated that the transgenic mouse model will reveal alterations in phenomena such as hyperexcitability, wind-up, and allodynia. This will require electrophysiological studies that are presently under way. As for the effects on motor activity, it has been previously shown that central injections of SP induce locomotion activity, probably owing to activation of the mesolimbic pathway via increased release and metabolism of dopamine (Kelley and Iversen, 1979; Kelley et al., 1979; Naranjo et al., 1984). Consistently, transgenic mice expressing anti-SP antibodies show a decreased locomotion activity and exploration behavior. Further investigations are needed to clarify whether this can be explained by changes in the SP-mediated dopamine release in the nucleus accumbens, and to determine the precise site of action of the antibodies in causing these motor deficits. It should be noted that, for practical reasons, the behavioral assays described in this paper were performed at an age (3 months) in which the levels of antibodies are rather low and show a high degree of interindividual variability. Thus, these effects may have been underscored.

The immunological consequences of the ectopically expressed transgenic antibodies are of great interest and are being investigated. Our data show that the serum of both families (family 8510 and 8) does not contain mouse antibodies directed against the human transgenic ones. This shows that transgenic antibodies, being present in serum from the early postnatal period, are tolerated by the immune system, as demonstrated in other transgenic models (Miller et al., 1989; Goodnow et al., 1990).

It is in principle possible that the secretion of antibodies in the nervous system of transgenic mice results in some nonspecific effect. However, this is unlikely, as previous work has shown that neuronal and glial cells secrete recombinant antibodies very efficiently, with no sign of suffering (Cattaneo and Neuberger, 1987). In the present transgenic model, the interaction between the antibody and the neuropeptide is most likely to occur in the extracellular space, as there is no chance for intracellular interaction: antibodies are secreted through the constitutive secretory pathway, while the neuropeptide is stored in granules of the regulated exocytic pathway. A direct demonstration of the interference of the antibodies with the

synaptically released SP peptide will be provided by electrophysiological studies of the synaptic transmission in these mice.

The successful application of the neuroantibody approach (Cattaneo and Neuberger, 1987; Piccioli et al., 1991) to interfere in vivo with the action of a target neuronal antigen confirms this approach as a complement to that of gene knockout by homologous recombination. It is likely that an important application of this experimental strategy will be in the study of the adult nervous system, with the transgenic antibody being kept transcriptionally silent. Thus, the neuroantibody approach allows the creation of different experimental models for the same target antigen of interest, in which the spatiotemporal expression patterns of the transgenic antibody can differ according to the promoter utilized for the transcriptional control of the transgene. In the case of the *vgf* promoter described above, the expression of the antibody is mainly postnatal and predominantly restricted to the CNS, while the antibody levels found in the serum are rather low and most probably reflect the secretion of antibodies from cells of neuroendocrine origin, or from cells of the adrenal medulla. The overall phenotype observed in the *vgf* anti-SP mice is rather mild, probably as the antibodies are mainly acting postnatally. It will be interesting to compare it with that obtained in mice in which the same antibody is expressed earlier in development (A. C. and P. P., unpublished data).

The range of proteins that can be studied by this approach is not limited to those acting extracellularly, as recombinant antibodies have been successfully targeted to different intracellular compartments, where they can compete with the corresponding antigen (Biocca and Cattaneo, 1995). As further applications of the neuroantibody approach are pursued (Ruberti et al., 1993), it will be possible to evaluate further the power and limitations of this antibody-based phenotypic knockout for functional studies in the developing and adult nervous system.

Experimental Procedures

Production of Transgenic Mice

The plasmids pSVhygro-VGF8aV$_\kappa$SP and pSVgpt-VGF8aV$_H$SP, carrying the light and heavy chain genes of the chimeric antibody NC1/34HL (Piccioli et al., 1991) under the transcriptional control of the NGF-inducible rat gene *vgf* (Levi et al., 1985), were digested at completion with PvuI and HindIII–BglII, respectively, to isolate the transcriptional unit. While the transcriptional unit for the heavy chain did not include vector sequences, that for the light chain did, alongside two enhancer sequences from immunoglobulin genes (see Figure 1). The 8.9 and 5.1 kb fragments containing the transcriptional units for the light and heavy chains, respectively, were electrophoresed in 0.7% low melting point agarose and then purified by NACS 52-PREPAC column (BRL). A total of 1–2 pl of each of the purified DNA samples (1–2 ng/ml) was coinjected in the male pronucleus of 1-cell fertilized eggs from F1 (C57BL/6J × CBaF1), and 12–24 hr later, injected eggs were reintroduced in the oviduct of pseudopregnant foster females F1 (C57BL/6J × CBA/Ca), as described (Allen et al., 1987). Family 8 mice were obtained in a set of separate experiments using the plasmids based on pSVhygro-IgV$_\kappa$SP and pSVgpt-IgV$_H$SP (Piccioli et al., 1991). Microinjections were performed in C57BL/6 × SJLF2 hybrid mouse eggs (DNX Corp.)

Analysis of *vgf* anti-SP mice was performed by Southern blot and dot blot. Briefly, 20 μg of genomic DNA from tail biopsies was digested with BamHI using ^{32}P-labeled DNA fragments encompassing human constant regions as probes. For the heavy chain, a BamHI–BglII fragment was purified from the vector pSVgpt-VGF8aV$_H$SP, while for the light chain we used a SacI fragment from plasmid pSVhygro-VGF8aV$_\kappa$SP. Probes labeled with ^{32}P to a high specific activity by random priming were used under high stringency hybridization (50% formamide, 6 × SSC, 5 × Denhardt's, 0.5% SDS, 0.2 mg/ml sonicated salmon sperm DNA at 42°C) and washing (0.2 × SSC, 0.1% SDS at 65°C) conditions.

RNA Analysis

Anesthetized mice were sacrificed, and tissues were dissected, immediately frozen in liquid nitrogen, and stored at −70°C until use. Total RNA was isolated following the guanidine-isothiocyanate procedure (Chomcynzski and Sacchi, 1987) and analyzed by RNase protection assay, using the following probes: V$_H$ (PstI–BamHI; 555 bp) from M13-IgP-V$_H$SP (Piccioli et al., 1991) cloned in Bluescript KS(+) and cut with PstI–BamHI; and V$_\kappa$ (PvuII–BamHI; 466 bp) from M13-IgP-V$_\kappa$SP (Piccioli et al., 1991) cloned in Bluescript KS(+) and cut with SmaI–BamHI.

PvuII fragments from these vectors (983 and 903 bp, respectively) were used as templates for RNA transcription with T7 polymerase in the presence of [^{32}P]UTP (Amersham; 800 Ci/mmol). The size of the expected protected band is 345 bp for V$_H$ and 309 bp for V$_\kappa$.

The probe for *vgf* mRNA was a BamHI–SphI, 580 bp fragment (corresponding to amino acids 443–635 of the VGF protein, in the 3′ region of the rat *vgf* gene, which is most conserved across species) in pGEM4Z (kindly provided by R. Possenti). The RNA probe was transcribed with T7 polymerase.

V$_\kappa$, V$_H$, and *vgf* antisense RNA probes were hybridized (or cohybridized) to 20 μg of RNA in 80% formamide (46°C for 12–18 hr) and treated with RNase A and T1. Protected RNA fragments were electrophoresed through a 4%–6% acrylammide, 8 M urea gel and autoradiographed.

SP and Anti-SP Antibody Measurements in the Serum and in Tissues

SP levels were determined essentially as in Donnerer et al. (1992). Briefly, the tissue samples from brain were placed in 20 vol of 2 N acetic acid, heated three times for 10 s each in a microwave oven to destroy peptidase activity, and homogenized by sonication. Homogenates were centrifuged, and the supernatants were freeze dried. SP immunoreactivity on the reconstituted samples was determined using rabbit polyclonal antibody RD2 (S. Leeman) and ^{125}I-labeled Bolton-Hunter-SP (Amersham) as tracer.

For the determination of human anti-SP antibodies in the serum by ELISA, transgenic and wild-type mice were bled from the retro-orbital sinus starting from P16, whereas younger animals were sacrificed and blood was collected transcardially under slight anesthesia conditions. Serum (50 μl) from each separate mouse was tested by ELISA on plates coated with SP conjugated to bovine serum albumin (SP–BSA; 5 mg/ml). Specifically, bound human/rat anti-SP antibodies were detected using a 1:750 dilution of horseradish peroxidase–linked goat anti–human immunoglobulin antibodies (Amersham) and developed with 1-Step Turbo TMB ELISA (Pierce). The presence of mouse anti-human antibodies was detected using biotinylated goat anti-mouse antibodies at a 1:2000 dilution (Vector) followed by a 1:500 dilution of streptavidin–horseradish peroxidase (Amersham). For the determination of ^{125}I–SP-binding antibodies in the blood, 100 μl of serum was preincubated for 5 hr at 4°C in radioimmunoassay buffer (8 mM NaH_2PO_4, 43 mM Na_2HPO_4, 3 mM NaN_3, 145 mM NaCl, 0.1% albumin, 0.1% gelatin, 0.1% Triton X-100); 15–20 fmol of ^{125}I–SP was then added, and the reaction was left to react for 36 hr at 4°C. Labeled SP was separated by the addition of 2 vol of charcoal/dextran T70 separation buffer (9.5 mM NaH_2PO_4, 40 mM Na_2HPO_4, 3 mM NaN_3, 145 mM NaCl, 0.625% charcoal/0.0625% dextran T70). The bound fraction (supernatant) was separated 30 min later by centrifugation at 2000 × g for 15 min, and the radioactivity was counted. Tissue extracts were prepared from brain regions of 20-day- and 3-month-old mice (single-transgenic, double-transgenic, and controls). Tissue (0.1–0.5 g) was extracted in 1–2 ml of ice-cold homogenization buffer (125 NaCl, 50 mM Tris, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40 [pH 7.4], and protease inhibitors [1 mM phenylmethylsulfonyl fluoride, 100

μg/ml aprotinin, 100 μg/ml leupeptin, 20 μg/ml chymostatin]) with a Polytron homogenizer. The homogenate was then subjected to a low speed centrifugation, and the soluble fraction was separated by a high speed centrifugation (100,000 × g for 30 min). The protein content of the S100 fraction was determined by the Lowry method, before antibody purification on a protein A–Sepharose column. Eluted antibodies were then challenged with 15–20 fmol per experimental point of ^{125}I-labeled Bolton Hunter-SP (Amersham), and the amount of antibody-bound SP was determined in duplicates, as described above. Standard calibration curves were run in each experiment, with serial dilutions of the affinity-purified NC1/34HL antibody. The background values obtained for nontransgenic controls and for the light chain transgenic mice were always between 0.4% and 4%.

Immunocytochemistry

Mice were anesthetized with Nembutal (0.1 ml per 100 g of body weight) and transcardially perfused with 4% paraformaldehyde in PBS. Brains and spinal cord were removed and postfixed overnight, cryoprotected in 20% sucrose in PBS, frozen in dry ice, and stored at −70°C until cutting. Cryostat sections (40–50 μm) were incubated in 0.003% H_2O_2, 10% fetal calf serum (FCS) in PBS before being analyzed as floating sections for the presence of transgenic antibody chains using the avidin-biotin-peroxidase Elite Standard kit (Vector Laboratories). Biotinylated anti-human IgG (Amersham) at 1:500 (0.1% Triton X-100, 10% FCS in PBS) and anti-human light chain chain (Amersham and/or Vector) at 1:50–1:100 were used as primary antibodies. Sections from age-matched control mice were also analyzed in parallel under the same conditions, in addition to sections in which primary antibodies were omitted.

Functional and Behavioral Experiments

The *vgf* anti-SP transgenic mice were used for the experiments at the age of 2–3 months. Basically, the same animals were used for all three functional tests, in the following order: open field tests, nociceptive tests, and neurogenic inflammation tests, with a 1 week interval between the individual tests. Family 8 mice have been systematically tested for locomotory activity and neurogenic inflammation tests, and in a preliminar way for acute nociception.

For the hot plate test, mice were placed on a 55°C hot aluminium plate 19 cm in diameter with a 20 cm high perspex cylinder. Licking of the hindpaws or jumping was used as endpoint. For the tail withdrawal test, mice were held in tubular restraining cages, and the terminal two-thirds of the tail were dipped into water at 50°C. In the open field test, mice were placed in a square box of 80 × 80 × 40 cm. A grid of black lines 10 cm apart was painted on the floor. The box was illuminated with a 60 W bulb positioned 80 cm above the floor. The mice were videotaped, and the following activities were counted for 10 min: locomotor activity (number of lines crossed) and rearing. The box was cleaned with alcohol after each animal. The data were collected from animals entering the apparatus for the first time. In the test for mustard oil–induced neurogenic inflammation, mice were anesthesized with sodium pentobarbitone (50 mg/kg, intraperitoneally), and the jugular vein was exposed to inject Evans blue (30 mg/kg). After a 5 min waiting period, mustard oil (5% in paraffin oil, 15 min contact time) was applied on the dorsal skin of one hindpaw, and the contralateral paw was used as a control (paraffin oil application alone). The level of extravasated protein-bound Evans blue in the paw was determined (620 nm) after formamide extraction. Data were analyzed using the Mann–Whitney test.

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